

## **Supplementary File**

### **Probiotic reduces bacterial translocation in type 2 diabetes mellitus: A randomised controlled study.**

Junko Sato<sup>1</sup>, Akio Kanazawa<sup>1,2</sup>, Kosuke Azuma<sup>1</sup>, Fuki Ikeda<sup>1</sup>, Hiromasa Goto<sup>1</sup>, Koji Komiya<sup>1</sup>, Rei Kanno<sup>1</sup>, Yoshifumi Tamura<sup>1,4</sup>, Takashi Asahara<sup>5,6</sup>, Takuya Takahashi<sup>5,6</sup>, Koji Nomoto<sup>5,6</sup>, Yuichiro Yamashiro<sup>5</sup>, Hirotaka Watada<sup>1,2,3,4</sup>

1) Department of Metabolism & Endocrinology, 2) Center for Therapeutic Innovations in Diabetes, 3) Center for Identification of Diabetic Therapeutic Targets, 4) Sportology Center, 5) Probiotics Research Laboratory, Juntendo University Graduate School of Medicine, 6) Yakult Central Institute

**S1 Table Primers used in this study**

Target bacteria*	Primer	Sequence (5' - 3')	Ref
<i>Clostridium coccoides</i> group	g-Ccoc-F	AAATGACGGTACCTGACTAA	6
	g-Ccoc-R	CTTGAGTTTCATTCTTGCAGA	
<i>Clostridium leptum</i> subgroup	sg-Clept-F	GCACAAGCAGTGGAGT	7
	sg-Clept-R3	CTTCCTCCGTTTGTCAA	
<i>Bacteroides fragilis</i> group	g-Bfra-F2	AYAGCCTTCGAAAGRAAGAT	8
	g-Bfra-R	CCAGTATCAACTGCAATTAA	6
<i>Bifidobacterium</i>	g-Bifid-F	CTCCTGGAAACGGGTGG	6
	g-Bifid-R	GGTGTCTTCCCAGATATCTACA	
<i>Atopobium</i> cluster	g-Atopo-F	GGGTTGAGAGACCGACC	7
	g-Atopo-R	CGGRGCTTCTTCTGCAGG	
<i>Prevotella</i>	g-Prevo-F	CACRGTAACGATGGATGCC	6
	g-Prevo-R	GGTCGGGTTGCAGACC	
<i>Akkermansia muciniphila</i>	AM1	CAGCACGTGAAGGTGGGGAC	9
	AM2	CCTTGCCTTGGCTTCAGAT	
<i>Clostridium difficile</i>	Cd-lsu-F	GGGAGCTTCCCATACTGGTTG	4
	Cd-lsu-R	TTGACTGCCTCAATGCTTGGC	
<i>Clostridium perfringens</i>	s-Clper-F	GGGGGTTTCAACACCTCC	2
	CIPER-R	GCAAGGGATGTCAAGTGT	5
<i>Lactobacillus gasseri</i> subgroup	sg-Lgas-F	GATGCATAGCCGAGTTGAGAGACTGAT	2
	sg-Lgas-R	TAAAGGCCAGTTACTACCTCTATCC	
<i>Lactobacillus brevis</i>	s-Lbre-F	ATTTGTTGAAAGGTGGCTTCGG	2
	s-Lbre-R	ACCCTTGAACAGTTACTCTCAAAGG	
<i>Lactobacillus casei</i> subgroup	sg-Lcas-F	ACCGCATGGTTCTTGGC	2
	sg-Lcas-R	CCGACAACAGTTACTCTGCC	
<i>Lactobacillus fermentum</i>	LFer-1	CCTGATTGATTGGTCGCCAAC	2
	LFer-2	ACGTATGAACAGTTACTCTACAGT	
<i>Lactobacillus fructiborans</i>	s-Lfru-F	TGCGCCAATGATAGTTGA	2
	s-Lfru-R	GATACCGTCGCGACGTGAG	
<i>Lactobacillus plantarum</i> subgroup	sg-Lpla-F	CTCTGGTATTGATTGGTGCTTCAT	2
	sg-Lpla-R	GTTGCCACTCACTCAAATGTAAA	
<i>Lactobacillus reuteri</i> subgroup	sg-Lreu-F	GAACGCAYGGCCAA	2
	sg-Lreu-R	TCCATTGTGGCCGATCAGT	
<i>Lactobacillus ruminis</i> subgroup	sg-Lrum-F	CACCGAATGCTGCAYTCACC	2
	sg-Lrum-R	GCCGCGGGTCCATCCAAA	
<i>Lactobacillus sakei</i> subgroup	sg-Lsak-F	CATAAAACCTAMACCCGATGG	2
	sg-Lsak-R	TCAGTTACTATCAGATACRTTCTC	
<i>Enterobacteriaceae</i>	En-lsu-3F	TGCCGTAACCTCGGGAGAAGGCA	1
	En-lsu-3'R	TCAAGGACCACTGTTCACTGTC	
<i>Enterococcus</i>	g-Encoc-F	ATCAGAGGGGATAACACTT	2
	g-Encoc-R	ACTCTCATCCTTGTCTTCTC	
<i>Streptococcus</i>	g-Str-F	AGCTTAGAAGCAGCTATTCAITC	3
	g-Str-R	GGATACACCTTCGGTCTCTC	
<i>Staphylococcus</i>	g-Staph-F	TTTGGGCTACACACGTGCTACAATGGACAA	2
	g-Staph-R	AACAACTTTATGGGATTGCWTGA	
<i>Pseudomonas</i>	PSD7F	CAAAACTACTGAGCTAGAGTACG	1
	PSD7R	TAAGATCTCAAGGATCCCAACGGCT	
<i>L.casei</i> strain Shirota <sup>\$</sup>	pLcS-57F	CTCAAAGCCGTGACGGTC	10
	pLcS-597R	ACGTGGTGCTAATAATCCTAGTG	

\* Group-, genus- or species specific primer sets were developed by using 16S rDNA sequences, except for Cd-lsu-F/R, En-lsu-3F/3'R, and g-Str-F/R, which targeted 23S rDNA.

<sup>\$</sup> Strain-specific primers sets for *L. casei* strain Shirota were developed by using the LcS-specific sequences (DDBJ/GenBank/EMBL accession number: AB246299).

## References

1. Matsuda K, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. *Appl Environ Microbiol* 2007;73: 32-39.
2. Matsuda K, Tsuji H, Asahara T, Matsumoto K, Takada T, Nomoto K. Establishment of an analytical system for the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of multicopy rRNA molecules. *Appl Environ Microbiol* 2009;75: 1961-1969.
3. Sakaguchi S, Saito M, Tsuji H, Asahara T, Takata O, Fujimura J, et al. Bacterial rRNA-targeted reverse transcription-PCR used to identify pathogens responsible for fever with neutropenia. *J Clin Microbiol* 2010;48: 1624-1628.
4. Matsuda K, Tsuji H, Asahara T, Takahashi T, Kubota H, Nagata S, et al. Sensitive quantification of *Clostridium difficile* cells by reverse transcription-quantitative PCR targeting rRNA molecules. *Appl Environ Microbiol* 2012;78: 5111-5118.
5. Kikuchi E, Miyamoto Y, Narushima S, Itoh K. Design of species specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. *Microbiol Immunol* 2002;46: 353–358.
6. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol* 2002;68: 5445-5451.
7. Matsuki T, Watanabe K, Fujimoto J, Takeda T, Tanaka R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* 2004;70: 7220-7228.
8. Matsuki T. Development of quantitative PCR detection method with 16S rRNA gene-targeted genus- and species-specific primers for the analysis of human intestinal microflora and its application. *Nihon Saikin Gaku Zasshi* 2007;62: 255-261. [Article in Japanese]
9. Derrien M. Mucin utilisation and host interactions of the novel intestinal microbe *Akkermansia muciniphila*. Ph.D. thesis (ISBN 978-90-8504-644-8). Wageningen University, Wageningen, The Netherlands, 2007.
10. Fujimoto J, Matsuki T, Sasamoto M, Tomii Y, Watanabe K. Identification and quantification of *Lactobacillus casei* strain Shirota in human feces with strain-specific primers derived from randomly amplified polymorphic DNA. *Int J Food Microbiol* 2008;126: 210-215.

**S2 Table Groups comparison of fecal microbiota at 8 and 16 weeks by linear mixed model analysis**

	8 weeks		16 weeks		Differences in least mean square (95% CI)
	Control	Probiotic	Control	Probiotic	
Total bacteria	10.2 (0.1)	10.3 (0.1)	10.2 (0.1)	10.4 (0.1)	0.15 (-0.09, 0.38)
Obligate anaerobes					
<i>C. coccoides</i> group	9.4 (0.1)	9.6 (0.1)	9.5 (0.1)	9.8 (0.1) *	0.25 (0.02, 0.47) *
<i>C. leptum</i> subgroup	9.5 (0.1)	9.5 (0.1)	9.4 (0.1)	9.7 (0.1) *	0.31 (0.02, 0.59) *
<i>Bacteroides fragilis</i> group	8.6 (0.1)	8.6 (0.1)	8.7 (0.1)	8.8 (0.1)	0.08 (-0.24, 0.39)
<i>Bifidobacterium</i>	9.0 (0.1)	8.8 (0.1)	9.0 (0.1)	8.9 (0.1)	-0.14 (-0.53, 0.26)
<i>Atopobium</i> cluster	9.4 (0.1)	9.3 (0.1)	9.4 (0.1)	9.5 (0.1)	0.03 (-0.19, 0.25)
<i>Prevotella</i>	8.3 (0.3)	8.2 (0.2)	8.7 (0.2)	8.6 (0.2)	-0.19 (-0.72, 0.34)
<i>Akkermansia muciniphila</i>	7.9 (0.2)	7.5 (0.3)	7.8 (0.3)	7.6 (0.3)	-0.14 (-0.88, 0.59)
<i>C. difficile</i>	NA	NA	NA	NA	NA
<i>C. perfringens</i>	3.7 (0.9)	5.8 (0.9)	5.3 (0.6)	4.6 (0.6)	-0.72 (-2.58, 1.14)
Facultative anaerobes					
Total <i>Lactobacillus</i>	6.1 (0.2)	7.7 (0.2)**	6.6 (0.2)	7.8 (0.2) **	1.17 (0.66, 1.68) **
<i>L. gasseri</i> subgroup	5.6 (0.2)	5.8 (0.2)	5.9 (0.2)	6.0 (0.2)	0.07 (-0.52, 0.66)
<i>L. brevis</i>	3.8 (0.3)	4.6 (0.5)	4.2 (0.7)	5.5 (2.2)	1.31 (-4.00, 6.61)
<i>L. casei</i> subgroup	4.8 (0.3)	7.4 (0.2) **	5.5 (0.3)	7.5 (0.2) **	1.95 (1.20, 2.69) **
<i>L. fermentum</i>	6.8 (0.3)	6.0 (0.3)	6.9 (0.2)	6.5 (0.2)	-0.40 (-1.03, 0.24)
<i>L. fructivorans</i>	NA	NA	NA	NA	NA
<i>L. plantarum</i> subgroup	4.3 (0.2)	4.3 (0.2)	4.4 (0.3)	4.3 (0.3)	-0.05 (-0.86, 0.76)
<i>L. reuteri</i> subgroup	5.3 (0.2)	5.3 (0.2)	5.5 (0.2)	5.5 (0.2)	-0.02 (-0.53, 0.49)
<i>L. ruminis</i> subgroup	5.8 (0.3)	5.6 (0.3)	5.4 (0.3)	5.9 (0.3)	0.47 (-0.36, 1.30)
<i>L. sakei</i> subgroup	5.3 (0.4)	6.0 (0.9)	4.8 (0.4)	4.0 (0.6)	-0.80 (-2.45, 0.84)
<i>Enterobacteriaceae</i>	6.9 (0.2)	7.0 (0.2)	6.8 (0.2)	7.0 (0.2)	0.19 (-0.35, 0.73)
<i>Enterococcus</i>	6.2 (0.2)	6.3 (0.2)	6.0 (0.2)	6.5 (0.2)	0.53 (-0.15, 1.20)
<i>Streptococcus</i>	8.3 (0.1)	8.3 (0.1)	8.5 (0.2)	8.5 (0.1)	-0.02 (-0.44, 0.41)
<i>Staphylococcus</i>	4.4 (0.1)	4.4 (0.1)	4.5 (0.2)	4.4 (0.1)	-0.11 (-0.52, 0.30)
Aerobes					
<i>Pseudomonas</i>	4.0 (0.7)	4.7 (0.6)	4.0 (0.8)	4.5 (0.5)	0.52 (-2.11, 3.15)
Administration of <i>L. casei</i> strain Shirota	NA	7.5 (0.0)	NA	8.1 (0.0)	NA

\* $p < 0.05$  vs. Control, \*\* $p < 0.01$  vs. Control. The results are expressed as least mean square (SE) ( $\log_{10}$

cells/g of feces). NA: Not available, CI: Confidence interval

**S3 Table Groups comparison of fecal organic acids and pH at 8 and 16 weeks by linear mixed model analysis**

	8 weeks		16 weeks		Differences in least mean square (95% CI)
	Control	Probiotic	Control	Probiotic	
Total organic acids	95.6 (5.5)	93.7 (5.5)	111.5 (5.7)	97.8 (5.7)	-13.67 (-29.97, 2.63)
Acetic acid	54.9 (3.4)	56.2 (3.4)	61.2 (3.3)	55.0 (3.3)	-6.18 (-15.59, 3.22)
Propionic acid	20.0 (1.4)	20.4 (1.4)	24.2 (1.6)	21.3 (1.6)	-2.92 (-7.39, 1.55)
Butyric acid	13.5 (1.4)	11.5 (1.3)	16.9 (1.7)	13.5 (1.5)	-3.48 (-8.04, 1.09)
Isovaleric acid	4.2 (0.6)	3.6 (0.6)	5.6 (0.8)	4.0 (0.7)	-1.65 (-3.75, 0.45)
Valeric acid	3.1 (0.4)	2.2 (0.3)	5.1 (0.8)	2.9 (0.7) *	-2.26 (-4.34, -0.17) *
Succinic acid	3.3 (1.0)	1.4 (1.0)	2.6 (3.3)	7.3 (3.0)	4.71 (-4.38, 13.81)
Formic acid	1.7 (0.4)	1.2 (0.4)	2.1 (0.4)	1.1 (0.4)	-1.03 (-2.19, 0.13)
Lactic acid	NA	NA	NA	NA	NA
pH	6.7 (0.1)	6.8 (0.1)	6.6 (0.1)	6.7 (0.1)	0.15 (-0.06, 0.36)

\* $p < 0.05$  vs. Control, The results are expressed as least mean square (SE) ( $\mu\text{mol/g}$  of feces). NA: Not available, CI: Confidence interval

available, CI: Confidence interval

**S4 Table Groups comparison of clinical parameters at 8 and 16 weeks by linear mixed model analysis**

	8 weeks		16 weeks		Differences in least mean square (95% CI)
	Control	Probiotic	Control	Probiotic	
BMI (kg/m <sup>2</sup> )	24.1 (0.1)	24.0 (0.1)	24.1 (0.1)	24.1 (0.1)	0.02 (-0.39, 0.43)
HbA1c (%)	6.9 (0.0)	6.9 (0.0)	6.9 (0.1)	7.0 (0.1)	0.08 (-0.09, 0.25)
Glycoalbumin (%)	17.6 (0.2)	17.7 (0.2)	17.6 (0.2)	17.9 (0.2)	0.32 (-0.37, 1.01)
Fasting blood glucose (mg/dL)	129.8 (2.8)	132.1 (2.8)	136.6 (3.2)	133.5 (3.2)	-3.12 (-12.17, 5.92)
Fasting C-peptide (ng/ml)	1.8 (0.1)	1.9 (0.1)	1.8 (0.1)	1.8 (0.1)	0.03 (-0.17, 0.24)
T-CHO (mg/dL)	190.9 (3.1)	192.3 (3.1)	191.4 (2.6)	189.7 (2.6)	-1.70 (-9.10, 5.70)
HDL-C (mg/dL)	57.1 (0.9)	54.6 (0.8) *	55.9 (1.2)	54.8 (1.2)	-1.12 (-4.51, 2.28)
TG (mg/dL)	113.1 (8.8)	119.7 (8.7)	121.1 (11.7)	108.7 (11.5)	-12.41 (-45.11, 20.30)
hs-CRP (mg/dL)	814.7 (1187.6)	2631.8 (1187.6)	1238.4 (526.1)	1551.2 (526.1)	312.78 (-1180.52, 1806.08)
TNF-α (pg/mL)	0.9 (0.1)	1.1 (0.1)	1.0 (0.1)	1.1 (0.1)	0.09 (-0.11, 0.29)
IL-6 (pg/mL)	1.9 (0.2)	1.8 (0.2)	2.0 (0.3)	2.1 (0.3)	0.15 (-0.80, 1.10)
Adiponectin (μg/mL)	9.0 (0.2)	9.3 (0.2)	9.2 (0.4)	9.4 (0.4)	0.23 (-0.96, 1.43)
LBP (μg/mL)	10.5 (0.7)	11.4 (0.7)	10.4 (0.6)	10.3 (0.6)	-0.18 (-1.80, 1.44)

\**p* < 0.05 vs. Control, The results are expressed as least mean square (SE), CI: Confidence interval

See Table 1 for abbreviations.